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(54) Title: KARYOTYPING MEANS AND METHOD

(57) Abstract

A method of comparing at least one chromosome or part thereof from a cell of an individual with an unknown kyryotype with the corresponding chromosome or part thereof of an individual with a known karyotype. The method includes the steps of: (a) obtaining DNA derived from a single cell of the unknown karyotype; (b) amplifying the chromosomal DNA of the unknown karyotype sufficiently for comparative purposes: (c) labelling the amplifed unknown chromosomal DNA with a first label and labelling a known chromosomal DNA with a second label, the first and second label being detectably different; (d) hybridising the labelled known chromosomal DNA with a chromosomal spread, and hybridising the labelled and amplified unknown chromosomal DNA with the chromosomal spread; and (e) forming an image of the chromosomal spread, and comparing the relative amount of first and second label as a function of position on the at least one chromosome or part thereof. It is also possible in part (b) to add extra primers to highlight specific loci of interest. The method may be used for preimplantation diagnosis (PGD) from a single cell source.

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KARYOTYPING MEANS AND METHOD

This invention relates to determining differences in chromosomes between individuals.

BACKGROUND TO THE INVENTION

Genetic screening for abnormalities that give rise to adverse health consequences is a well established procedure that may be used in prenatal diagnosis of abnormalities. The location of specific genes and/or diagnostic markers can be identified from portions of the genome that are present in abnormal copy numbers.

Using traditional analysis techniques, foetal cells needed to be cultured for several weeks to obtain enough cells for analysis. In more recent times techniques of *in situ* hybridisation that allow analysis of intact cell nuclei have been developed.

Polymerase chain reaction (PCR) amplification and fluorescent in situ hybridisation (FISH) have been successfully used for preimplantation diagnosis (PGD) in 20 centres around the world and more than one hundred unaffected children have been born following PDG (Verlinsky *et al*, 1997). However, most of these diagnoses use probes for specific loci and are therefore focused on one or a few specific loci or chromosome regions. These techniques therefore leave the majority of the genome unexamined and it is relatively costly and time consuming to test for numerous possible abnormalities. Chromosome preparation from preimplantation embryos has been reported (Sabtalo *et al*,1995), but accurately karyotyping in the blastomere has been proven to be extremely difficult (Harper, 1996).

Comparative genomic hybridisation (CGH) is a technique that has been used for determining the gains and losses of copy number of chromosomes or partial chromosomes from subject cells without chromosome preparation from test tissue (US patent 5665549 to Pinkel *et al*). CGH is now mainly used for the study of the genetics of cancer (Kallioniemi *et al* , 1996).

However, in the case of PGD the amount of sample material available from embryos or early foetuses is limited and for this reason it is not possible to prepare chromosome spreads from embryos or early foetuses. It is desirable however to extend CGH to be more readily applied to circumstances where small amounts of sample material are

available. A single cell contains less than 25 pg genomic DNA (Kuukasjarvi *et al*:, 1997) and thus substantially the whole genomic DNA from a single cell has to be amplified before it is used for a CGH test.

The technique of degenerate oligonucleotide primed (DOP)-PCR was designed to give general amplification of target DNAs at frequently occurring priming sites (Telenuis *et al.*, 1992). It is thought that DOP-PCR is able to amplify most of the genome equally (Ghaffari *et al* ,1998) and achieve several hundred-fold amplification of all sequences when there is sufficient starting template genomic DNA (Chueng and Nelson *et al.*, 1996). However, DOP-PCR with diluted genomic DNA at a single cell level has not yet been achieved (Kuukasjarvi *et al*, 1997) and DOP-PCR starting with DNA from a single cell has not been reported in the literature at the priority date of this application.

Extending the application of CGH to PGD has been suggested (Harper, 1996) however as with the study of tumour cells it is not possible to prepare chromosome spreads derived from embryos or early foetuses in a timely manner because there is simply not enough sample material. The prospects of achieving karyotyping using CGH and embryos or early foetuses was uncertain before the present invention because only samples of single or small numbers of cells are available. Further, prior to the present invention there was no available single cell technique for reliably covering substantially the entire genome, and clearly reliability is of importance when only small amounts of sample are available.

SUMMARY OF THE INVENTION

The present invention results from the finding that it is possible to reliably perform CGH karyotyping using DNA amplified from single cells.

Accordingly in a first aspect the invention could be said to reside in a method of comparing at least one chromosome or part thereof from a cell of an individual with an unknown karyotype with the corresponding chromosome or part thereof of an individual with a known karyotype, the method including the steps of :

a. obtaining DNA derived from a single cell of the unknown karyotype,
b. amplifying the chromosomal DNA of the unknown karyotype sufficiently for comparative purposes,

c. labelling the amplified unknown chromosomal DNA with a first label and labelling a known chromosomal DNA with a second label, the first and second label being detectably different,

d. hybridising the labelled known chromosomal DNA with a chromosomal spread, and hybridising the labelled and amplified unknown chromosomal DNA with the chromosomal spread, and

e. mapping the chromosomal spread, and comparing the relative amount of first and second label as a function of position on the at least one chromosome or part thereof.

Preferably the known chromosomal DNA is also obtained from a single cell source of known karyotype, and amplified before labelling in step c.

One of the preferred applications of this invention is in the prenatal diagnosis of chromosome differences for which application the source of the unknown single cells might thus be one of a number of types. In the case of an embryo it could be a single embryo cell which has been removed from a pre-implantation embryo by micromanipulation. It is known that normal births can result after cells are taken from such embryos. It will of course be understood that the amount of sample material taken from an embryo is very much limited. Other sources for prenatal testing might be oocyte, polar body, sperm cells or other somatic or germ cells.

In the case of a foetus, the sample might be a foetal blood cell taken from the maternal circulation or from the mothers reproductive tract (eg cervical or vaginal lavage). Foetal red blood cells, unlike mature blood cells, are nucleated and can be isolated in sufficient numbers from the maternal circulation to provide a reliable source of whole chromosomal DNA for testing purposes. This source is preferable to performing invasive procedures such as amniocentesis, which have a risk factor associated with them.

It is preferable to use a single cell as a source of the DNA of known karyotype and this may be chosen from a source easily collected such as white blood cells, but other sources might also readily be used. It has been found that a single cell of known karyotype may give clearer results and greater coverage of the genome than may be obtained using DNA from multiple cells and or other sources of DNA of known

karyotype. Without being bound by the same, it is postulated that the reason that a single cell source of the DNA of known karyotype gives clearer results, is that during amplification from a multiple cell source there may be a greater affinity of groups of primers to bind to a certain group of sites on the isolated DNA, and thus extension products from these groups of sites will predominate from the initial rounds of amplification, whereas where there are only single copy of all sites even those sites to which primers bind less readily will be amplified. It is thought therefore that there will be a different distribution of extension products if amplification commences from a single cell when compared to amplifications from a multiplicity of cells, several thousand or 10^6 cells, thereby introducing unnecessary inherent variation into the CGH test.

A similar effect may also be obtained using a small number of cells, and in particular if the number of cells of known and unknown are matched, perhaps to the same order of magnitude, and any DNA is isolated and amplified to approximately the same degree. It will be understood that using the technique of micromanipulation it is possible typically to isolate a single cell however it may be possible to obtain two or more cells and therefore the invention also contemplates the use of a small number of cells that may be obtained by micromanipulation. Thus, reference throughout this specification to a 'single cell' is to be understood to include a small number of cells, perhaps less 10 cells, and more preferably less than 5 cells.

Most preferably the known chromosomal DNA and the unknown chromosomal DNA are amplified by PCR using the same complement of primers for both. In this way the quality and quantity of the extension products resulting from the PCR reaction of both the known and unknown DNA are comparable. The results obtained using the present method are to be contrasted to those obtained if a tissue extract is used as a control and labelled, perhaps by nick translation, so that the DNA used is qualitatively different. The distribution of binding of primers to the chromosomes of the cell of known and unknown karyotypes means that after one round of extension by PCR the result is not two copies of the genome concerned, that is, the extension products resulting from PCR do not meet end to end to collectively form a complete genome. Rather there is a partial duplication, with some extension products being spaced apart quite considerably and there being overlaps with others. Where a different complement of primers is used for the known and unknown DNA or where the known is not amplified, and labelled

directly, perhaps by nick translation, a different labelling pattern will be introduced. This difference in labelling of chromosomal spreads is thought to be sufficiently problematic to detract from the sensitivity required for karyotyping using DNA amplified from single cells using the CGH technique.

In a preferred form the amplification of known and unknown DNA is by means of using degenerate primers, the technique sometimes being referred to as degenerate oligonucleotide primed (DOP)-PCR (Telenuis *et al*, 1992), which reference is incorporated herein in its entirety. This technique uses a family of primers, each with identical flanking sequences and a "random" central portion. Use of DOP-PCR generated extension products is known to give an adequate distribution of PCR extension products for purposes of CGH in tissue samples. The DOP-PCR technique can also be used to label the extension products appropriately by the incorporation of a labelled nucleotide into the reaction mix in the final round or rounds of amplification.

It will be understood that the choice of primer for the DOP-PCR need not be the commonly used degenerate universal primer but could also be any primer that amplifies a proportion of the genome sufficient to allow for the comparative analysis. Indeed other forms of so-called whole cell amplification might equally be used.

Other suitable techniques for amplification of the known and unknown DNA include primer-extension preamplication PCR (PEP-PCR; Zhang *et al*, 1992; incorporated herein in its entirety), ligation mediated PCR (Klein *et al*, 1999), tagged PCR (t-PCR; Grothues *et al*, 1993; incorporated herein in its entirety) or alu-PCR (Nelson *et al*, 1989; incorporated herein in its entirety).

In one preferred form of the invention the chromosomal spread is in the form of a metaphase spread slide from a known normal male. The mapping may involve forming an image of the chromosomal spread and comparing the relative amount of the first and second label as a function of position on the chromosome part thereof.

It is preferred that each known and unknown amplified and labelled DNA preparation is hybridised to more than one chromosome spread to minimise the effects of any lack of uniformity of hybridisation that might occur on any given metaphase spread.

According to the present invention, preferably greater than 90 % of the distributions of the standard deviations in autosomal regions fall within the range 0.85 - 1.15, the variation limits of the profile in a successful CGH (Kallioniemi *et al*, 1994). In contrast it was found that when genomic DNA was the reference material about 30 % of the distributions of the standard deviations in autosomal regions fell outside the range 0.85 - 1.15.

In another preferred form of the invention the chromosomal spread is in the form of an array of different nucleic acids representative of a complement of chromosomes and attached to the surface of a substrate (a 'gene chip'). Thus the labelled and amplified unknown and known chromosomal DNA may be hybridised with the biological plate of the gene chip, and the chromosomal spread mapped by interrogating arrays on the biological chip plate with a biological chip plate reader to generate assay results. Thus mapping of the chromosome spread may include forming an image with intensities in each region of the image varying according to the relative amount of first and second label. Techniques for the preparation and use of suitable biological chip plates can be found in US 5,874,219 to Rava *et al*, which is incorporated herein in its entirety. The use of automated gene chip technology provides a method for rapid CGH screening.

Certain regions of the chromosome cannot be reliably used for CGH, and these are generally referred to as heterochromatic or telomeric (end of chromosomes) regions. Often heterochromatin is associated with centromeres and thus centromeric regions cannot be analysed by CGH and these regions are discounted when comparisons of the binding between known and unknown karyotypes are made.

It will be understood that the invention preferably involves an assessment being made as to the number of copies of a chromosome(s) or portion thereof between individuals of known and unknown karyotype.

This technique is particularly useful in diagnosis of gross chromosomal differences and aneuploidy such as deletions, duplications or other amplifications. Conditions that might be amenable to detection by this technique include but are not limited to Trisomy 21, 13, 18 and detecting missing chromosome such as in Turner's syndrome (45, XO).

Whilst it will be understood that for the purpose of scanning for these general defects it is desirable to look at all chromosomes of the subject, it might under certain circumstances be desirable to look at only a partial karyotype, and the chromosome spread or gene chip need not necessarily include all chromosomes.

It will generally be desired to use normal male DNA as the DNA of known karyotype because of the presence of a Y chromosome.

Many variations to the invention involve the application of standard practices, and many of these variations are set out in US patent 5665549 to Pinkel *et al*, which reference is incorporated herein in its entirety.

In an alternative form the invention might be also be said to have resulted from the finding that the combination of DOP-PCR and CGH gives an effective result, whether a single cell source of DNA of unknown karyotype is used or not.

It is not anticipated that the CGH analysis will permit very fine analysis but only for rearrangements that are in the order of magnitude of mega bases, however, it may be possible to use the same sample of amplified DNA for testing for the presence or absence of specific alleles or defects in a gene. Thus a specific primer or primer group can be added to amplify the unknown DNA sample (and known sample) the primer or primer set might not permit amplification of a region of the chromosome with the specific defect or allele and then the absence of an allele can be probed for by a suitable probe, with suitable controls. Alternatively simply by ensuring that a primer is close to the region of interest will ensure that if the defect (or normal gene) is present it will be present in reliable numbers in the amplified DNA and then later probing by probes or techniques that can differentiate between the two alleles.

Some chromosomes or portions of chromosomes are difficult to analyse using CGH and it may be desired to highlight these chromosomes or portions of chromosomes. Thus, for example, chromosomes 21 and 22 are quite small in size and any chromosomal variation is relatively difficult to visualize especially in view of the fact that both these chromosomes have heterochromatic regions. It might be desirable to bias the primer complement in favour of highlighting certain regions, and to add primers specific for that region or chromosome. Thus highlight primers designed to

highlight certain chromosomes or portions of chromosomes might be added to the complement of primers. Those highlight primers might be made to bind to sequences known to be present in certain positions of the target for highlighting. Unique sequences might be determined from some of the marker present. Thus on chromosome 21 markers such as D21S131, and D21S142 and similar markers might be used, or in the alternative DNA sequences for any chromosome might be determined from the data resulting from the cooperative project for DNA sequencing of the entire human genome known as HUGO.

Accordingly in a second aspect the invention could be said to reside in a method of comparing at least one chromosome or part thereof from a cell of an individual with an unknown karyotype with the corresponding chromosome or part thereof of an individual with a known karyotype, the method including the steps of :

a. obtaining DNA derived from a single cell of the unknown karyotype,

b. amplifying the chromosomal DNA of the unknown karyotype sufficiently for comparative purposes using:

a degenerate primer for amplifying substantially the whole genome, and at least one specific highlight primer or primer group for amplifying specific loci or loci groups,

c. labelling the amplified unknown chromosomal DNA with a first label and labelling a known chromosomal DNA with a second label, the first and second label being detectably different,

d. hybridising the labelled known chromosomal DNA with a chromosomal spread, and hybridising the labelled and amplified unknown chromosomal DNA with the chromosomal spread,

e. mapping the chromosomal spread, and comparing the relative amount of first and second label as a function of position on the at least one chromosome or part thereof, and

f. analysing specific loci to determine allele type.

The method of the second aspect of the invention therefore allows for the detection of general chromosomal abnormalities as well as allele differences for specific loci.

Candidate genes to amplify with specific primers in addition to the general (DOP or similar) primers include disease loci of interest. For example, Caucasians have a high

incidence of cystic fibrosis (CF) therefore relevant primers for this locus could be included. Other disease loci for example include thalassemia, Duchenne muscular dystrophy, rare X-linked disorders, Haemophilia, Huntington's Disease. Indeed the molecular defect(s) behind single gene disorders and multiple gene disorders are being elucidated rapidly in this the genetic era. Therefore the number and type of primers added will increasingly reflect a specific mix determined to be beneficial to each family. As well as disease loci, other loci may also be amplified specifically such as polymorphic loci. This enables paternity/ maternity testing and the detection of contamination to increase the confidence in diagnosis.

Obtention and amplification of DNA derived from cells of known and unknown karyotype, labelling of the amplified unknown chromosomal DNA with a first label and labelling a known chromosomal DNA with a second label, hybridisation of the labelled known and unknown chromosomal DNA with the chromosomal spread, and comparing the relative amount of first and second label may be performed according to the first aspect of the invention.

It will be understood that this invention will also encompass a kit comprising reagents for performing the above methods.

BRIEF DESCRIPTION OF THE DRAWINGS

For a better understanding, the invention will now be described with reference to a number of examples which are also represented in the figures wherein,

Figure 1 is a photograph of various DNA samples eletrophoresed on an agarose gel and stained with ethidium bromide. The following were loaded in each lane:-
Lane 1: SSP-1 DNA/Eco RI fragment: 360-8510 bp,
Lane 2- 4: typical DOP-PCR products derived from 3 individual single cells,
Lane 5: Negative control,
Lane 6: Positive control,
Lane 7: DNA/Hind III fragment: 125 - 23,130 bp,
Lane 8: pUC19DNA/Hpa II fragment: 26 - 501 bp,

Figure 2 is a graphic representation printed by the Quips CGH Analysis software (Vysis) showing the results of the CGH of amplified DNA-SG (labelled with Spectrum Green) against normal genomic DNA-SR (labelled with Spectrum Red), for (a) XY vs XY and (b) XXY vs XY. For each CGH a graphic of the chromosome is represented to the left. The centromere of each chromosome is indicated by a constriction, the dark bands are representative of bands seen in staining of chromosomes and cross hatched portions represent heterochromatin portions of the chromosome concerned. A thick vertical line is some times present as a complete or broken line immediately adjacent to the graphic of the chromosome indicating when a region of one or other of the chromosomes has been calculated to have at least one further copy of a chromosome or region thereof. The vertically aligned graph to the right includes a central medial line, with darkened vertical lines to either side of the centre and a further three lines progressively further from the darkened lines. A calculation of the extent to which any region is increased or decreased in either the known or unknown chromosome is graphed and indicated by the dark graphed line, the standard deviation is also graphed in lighter lines on either side of the dark graphed lines. Once the dark graphed line breaks past the first of the vertical lines of the centre it is considered that an increase or decrease has occurred with respect to the known cell, and

Figure 3 is a representation similar to that of Figure 2 showing the results of the CGH of amplified DNA-SG (47 XY, +13) against amplified normal genomic DNA-TRITC (46, XY),

Figure 4 is a representation similar to that of Figure 3 showing the results of the CGH of amplified DNA-SG (47 XY, +21) against amplified normal genomic DNA-TRITC (46, XY),

Figure 5 is a representation of a microarray showing that a single cell tested relative to a known normal cell had extra copies of the terminal portion of chromosome 18 (as indicated by green fluorescence ), a deletion of the terminal portion of chromosome 14 (as indicated by red fluorescence) and normal chromosome 21 constitution (as indicated by yellow fluorescence), and

Figure 6 is a photograph of various DNA samples eletrophoresed on an agarose gel and stained with ethidium bromide. The following were loaded in each lane:-

Lane 1: phage Lambda/Hind III fragments: 125 - 23,130 bp,

Lane 2: pUC19/Hpa II fragments: 26-501 bp,

Lane 3: DOP-PCR products derived from an individual single cell amplified without the addition of specific primer for exon 10 of the cystic fibrosis gene,

Lane 4: DOP-PCR products derived from an individual single cell amplified with the addition of specific primer for exon 10 of the cystic fibrosis gene,

Note that the PCR products shown in lane 3 and 4 appear to be identical, especially with respect to intensity, size and distribution.

DETAILED DESCRIPTION OF EXAMPLES OF THE INVENTION.

EXAMPLE 1 - *Whole genome amplification and CGH using DOP-PCR and metaphase spread*

Materials and Methods

Single Cell DOP-PCR

A single cell each from an individual with the karyotype of 46,XY, 46,XX, 45,XO, 47,XXY, 47,XY,+13 and 47,XY,+21 were sorted into a 0.5 ml PCR tube under an inverted microscope. 5 µl of 200mM KOH, 50 mM DTT solution was added to each tube and heated at 65°C for 10 minutes for cell lysis. Then an equal amount (5µl) of 900mM Tris-HCl pH 8.3, 300 mM KCl, 200 mM HCl was added to neutralise the buffer.

For DOP-PCR, the following solutions were added into each tube, 2.5 µl of a 40 µM degenerate universal primer (5'-CCG ACT CGA GNN NNN NAT GTG G-3') (Boehringer Mannheim), 5 µl of 0.01% gelatin, 100 mM Tris-HCl pH 8.3, 5 µl of 2 mM each dNTP, 4 µl 25 mM KCl and 1 µl of Taq polymerase (Perkin-Elmer/Cetus, 5 units/µl). Final reaction volume was 50 µl.

DOP-PCR was run on a thermocycler (Minicyler, MJ Research) with the initial denaturation at 94 °C for 10 min, 5 cycles of random amplification: 94 °C for 1 min, 30 °C for 1.5 min, ramping from 30-72 °C for 3 min, 72 °C for 3 min, 35 cycles of non-random amplification: 94 °C for 1 min, 62 °C for 1 min, 72 °C for 3 min + 14 sec/cycle. The size of DOP-PCR product was checked by electrophoresis through a 1 % agarose gel. DOP-PCR products were purified using a High Pure PCR Purification Product kit (Boehringer Mannheim) and DNA concentration was estimated spectrophotometrically.

DNA labelling

DOP-PCR labelling: The amplified single cell DNA was labelled with SpectrumGreen (SG)-dUTP (Vysis) by another round of DOP-PCR. The reaction solution of labelling DOP-PCR (Ghaffari *et al*:, 1998) was comprised of 2 µl of amplified DOP-PCR products, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.0 mM $MgCl_2$, 0.001 % gelatine, 2 µM degenerated universal primer, 0.2 mM of dATP, dCTP, dGTP, 0.05 mM dTTP and 0.15 mM SpectrumGreen-dUTP (Vysis). 2.5 U of Taq polymerase. Final reaction

volume was 12.5 µl. PCR was run in 94 °C for 10 min, 30 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 3 min + 14 sec/cycle. DOP-PCR labelling efficiency was checked by electrophoresis of 5µl of products through a 1 % agarose gel.

Nick translation: Nick translation was performed in a 50 µl of reaction solution according to the method of Kirchhoff [Kirchhoff *et al.* (1998)]. The reaction mixture included 1 µg of DOP-PCR product, 5 µl of 10 x reaction buffer (500 mMTris-HCl pH 7.8, 50 mM $MgCl_2$, 100 mM 2-mercaptoethanol), 3 µl of 10 x enzyme mix [500 U/ml DNA polymerase I (Promega), 1 U/ml DNAse I (Promega), 100 mg/ml BSA, 50 mM Tris-HCl pH 7.5, 5mM magnesium acetate, 1 mM ß-mercaptoethanol, 50% (v/v) glycerol containing], 5µl of 0.2 mM each of dATP, dCTP, dGTP, 0.1 mM dTTP, 0.1 mM SpectrumGreen-dUTP (SP) (Vysis) or TRITC-dUTP (SP) (Boeheinger Mannheim). Incubate for 60 min at 15 °C and then 70 °C for 10 min. It should be visible as a smear between 100 and 1500 bp in after agarose gel electrophoresis. Otherwise, the concentration of enzyme mix and incubation time would be adjusted.

CGH

400 ng of SG (Spectrum Green) labelled DOP-PCR product amplified from the individual single cell (amplified DNA-SG), equal amount of SpectrumRed(SR) total human male genomic reference DNA (normal genomic DNA-SR) (Vysis) or TRITC-labelled DOP-PCR product amplified from normal male cell (normal amplified DNA-TRITC) and 10 mg of human Cot1 DNA (Promega) were combined and precipitated in 1/10 volume 3M sodium acetate and 2 volumes of 100% ethanol. After incubation at -20°C for 30 min, mixed DNA was centrifuged in 14,000 rpm for 30 min at 4°C. The supernatant was discarded and the pellet was dried for a few minutes at 60°C and dissolved in 10 µl 50% formamide, 10 % dextran sulphate, 2 x SSC, pH 7.0. The DNA was denatured at 70°C for 5 min just before hybridization.

A routinely prepared metaphase spread slide from a known normal male was dehydrated through a 70 %, 95 % and 100 % ethanol series. The slide was denatured in 70% deionized formamide, 2 x SSC, pH 7.0 at 70°C for 3 min. The denatured DNA mixture was applied onto the denatured chromosomes on the slide under a 18 x 18 mm coverslip sealed with rubber cement and incubated in a wet chamber at 37°C for 2-3 days. The posthybridization washing was carried out in 0.4 % NP40 (NONIDET®P40), 0.3 x SSC and 0.1 % NP40, 2 x SSC for 2 min each at 70 °C.

After air-drying, the preparation was counterstained with an antifade-mounting solution, 0.56 µg/ml DAPI in VectaShield (Vector Laboratories).

CGH images were captured by a CCD camera using the Quips XL Genetics Workstation (Vysis 30-143200) or Cytovision Ultr Image Collection System (Applied Imaging Int Ltd) and CGH analyses were carried out using Quips CGH Analysis Software (Vysis 30-143002).

Results

The yield of a single cell DOP-PCR reached at least 2 µg DNA as determined by spectrophotometric measurement after purification. The size of the amplified product ranged from 200 to 23,000 bp (predominantly : 400- 4000). The negative control always contained primer related products but their size was usually much shorter (about 1,000 bp) than single cell DOP-PCR product. The size of nick translation labelled DOP-PCR product (SG-amplified DNA or TRITC-amplified normal DNA) was 100 - 1,500 bp.

The data within centeromeric, telomeric and heterochromatic regions was generally ignored for analysis purposes.

The CGH results from the Image Analysis System (Vysis) showed that there were significant differences in the average profile of the green to red fluorescence ratio in the unknown amplified DNAs (labelled in Spectrum Green) compared to normal male genomic DNA. When genomic DNA was the reference material about 30 % of the distributions of the standard deviations in autosomal regions unexpectedly fell outside the range 0.85 - 1.15, the variation limits of the profile in a successful CGH (Kallioniemi *et al*, 1994). As shown in Figure 2 over representation of the X chromosome is clearly visible even though the unknown cell was a male cell the same as the genomic DNA reference. Therefore genomic DNA is not a reliable reference for single cell CGH.

For this reason we performed CGH using DNA amplified from a known normal single male cell as the reference material. It can therefore be seen that this is an important step in obtaining reliable and accurate CGH results on unknown single cells. When the amplified DNA from two single cells is compared the average ratio of the profile is very

close to 1.0. and the standard deviation is also close to the profile. This means that where no difference in chromosome copy number is expected, none is observed.

As shown in Figure 3 the profile for a cell (47, XY +13) containing an extra chromosome 13 clearly demonstrates an increase in the amount of Green fluorescence confirming the extra chromosome 13 material present in this unknown cell relative to the single male (known normal) cell reference DNA.

In the CGHs of amplified DNA-SG against amplified normal DNA-TRITC, the distributions of the standard deviations were usually close to the average profile. Only 6.3 % of the distribution fell outside the range of 0.85 -1.15. The profiles of all autosomes are very close to 1.0 in all CGHs where normal copy number of autosomes is expected. The profiles for the X and Y chromosomes were as expected when the following test cells were looked at: 46, XY; 45, XO; 46, XX; 47, XXY; 47, XY + 13; and 47, XY +21. Thus providing the correct diagnosis in each case.

As shown in Figure 4 the increase in green fluorescence for chromosome 21 when the test cell was 47, XY+21 is visible on the long arm of chromosome 21. This provides a correct diagnosis of trisomy 21 for this cell. As chromosome 21 is very small it may be necessary to further increase the sensitivity of CGH for this chromosome by adding some specific primers for markers on chromosome 21 to selectively enhance for this region.

An important feature of our DOP-PCR CGH protocol is the use of the same primer for amplifying both the unknown and the known single cell. This ensures, largely, that the same amplification products are produced from each cell. This makes comparison between the genomes much more sensitive. It is this increased sensitivity which allows the genomes of two single cells to be compared accurately and reliably.

Conclusion: Our single cell DOP-PCR method was able to offer enough amplified product of a suitable size range and profile for the purpose of CGH. Amplification does not appear to be uniform. Therefore a procedure that ensures that the same biases are present in the amplification products of unknown and known samples is very important. Two features of our CGH method perform this function namely the use of a

single cell as the reference (known) sample and the use of the same primer for amplification (in this case we have found the DOP-PCR primer to work very well).

EXAMPLE 2 - *Whole genome amplification and CGH using DOP-PCR and biological chip detection*

The traditional karyotype of an individual involves growing up around 10^7 cells from whole blood to give an actively dividing population of cells. The cells are then arrested in mitosis and spread onto a microscope slide to give a so called "metaphase spread". Metaphase chromosomes are sufficiently dense to be able to be seen and analysed by light microscopy. However as advances in DNA chip technology have now come to the stage that most researchers will have access to DNA microarrayers, it is no longer necessary to use traditional metaphase spreads for karyotyping. Instead an array of DNA (or RNA) clones or PCR products can be made on the traditional microscope slide. One, thousands and eventually hundreds of thousands of 2 nano litre spots (clones or PCR products) can be placed onto a single slide. These spots correlate to regions of the genomes in a defined manner such that it is known what position and along which chromosome the spot corresponds to in much the same way that a traditional karyotype does. It can be thought of that the old fashioned metaphase spread is an "analogue" version of a karyotype and that this is being replaced by DNA chips which are a "digital" version of a karyotype. The later being more amenable to automation and computer analysis.

There are a number of ways of obtaining the relevant clones or PCR products to place on a DNA chip. Clones can be bought, made from scratch or obtained from Whole Chromosome Paint (WCP) Probes which are commercially available (eg Vysis). To adapt WCP probes for this purpose it is necessary to isolate just certain of the individual probes within a WCP probe. This can be achieved by serial dilution or other means of picking clones both randomly and nonrandomly.

Once a series of clones along a given chromosome (and this would be extended to include all chromosomes) is produced onto a microscope slide, then the CGH hybridisation of PCR products labelled with two different labels (to distinguish the unknown from the known single cell sample) is performed in much the same way as to conventional metaphase spread. Changes to the protocol include taking into account the volume of probe required which depends on the number of "spots" on your DNA chip.

Once hybridisation has taken place the microscope is then placed into a "reader" which scans the microscope slide to determine fluorescence intensities (or other depending on the label used) in exactly the same way the QUIPS CGH software does. Instead of scanning chromosomes directly the reader scans each spot in turn in order to build up the array of information. The relative differences in intensities is then analysed and in this way the region (or regions) of the genome that are duplicated or deleted can be determined.

A typical result using a micro array is shown in Figure 5. This result indicates that the single cell tested here relative to a known normal cell had extra copies of the terminal portion of chromosome 18 (as indicated by green fluorescence), a deletion of the terminal portion of chromosome 14 (as indicated by red fluorescence) and normal chromosome 21 constitution (as indicated by yellow fluorescence).

An aliquot of the PCR reaction can be used to investigate specific loci of interest by other means not utilising gene chips or by a method that enables analysis by the same or a different gene chip (as appropriate) to that performing the CGH analysis.

EXAMPLE 3 - *Whole genome amplification using DOP-PCR and specific loci amplification for CGH*

The invention described here allows the "karyotypic" information to be combined with more conventional diagnoses for single gene disorders. In particular it allows the amplification with high efficiency of multiple other loci from a single cell to be combined with CGH analysis.

To the DOP (or similar) -PCR mastermix was added various amounts of single gene primer from exon 10 of the cystic fibrosis gene from approximately 1 pmol to 10 pmol. The DOP-PCR was then carried out as described earlier and an aliquot electrophoresed through an agarose gel. As can be seen from Figure 6 the DOP-PCR reaction occurred successfully without hindrance caused by the addition of small amounts of specific locus primer as determined by visual inspection. An aliquot was used to perform CGH as described above and the results were similar to that obtained without the addition of specific loci primers. This method can be extended to include other specific loci and in our hands up to 9 loci have been reliably amplified using the SEP analysis described below. Thus an aliquot of the DOP-PCR mix is used to perform the karyotype analysis

and other aliquots are used to perform each of the locus specific PCR analyses (or multiplexed as appropriate). Electrophoresis of the DNA samples showed the presence of the specific gene product of size approximately 155 bp from exon 10 of the CF gene demonstrates the ability to amplify specific loci as well as general loci.

Candidate genes to amplify with specific primers in addition to the general (DOP or similar) primers include disease loci of interest. For example cystic fibrosis (CF), thalassemia, Duchenne muscular dystrophy, rare X-linked disorders, Haemophilia, Huntington's Disease. Indeed any molecular marker which is determined to be a causative agent of a genetic disorder or merely to correlate with the disease in general or in that particular family can be used. Therefore the number and type of primers added will increasingly reflect a specific mix determined to be beneficial to each family. As well as disease loci it is also important to amplify other loci specifically such as polymorphic loci. This enables paternity/ maternity testing and the detection of contamination to give increased confidence in diagnosis.

EXAMPLE 4 - *Whole genome amplification using PEP (primer extension preamplification) and specific loci amplification*

Whole genome amplification using general primers other than DOP-PCR with the addition of specific primers works very well on single cells. We have called the addition of specific primers to primer extension preamplification (PEP) primers: Selectively Enhanced Primer extension preamplification or SEP. We have successfully added in specific primers for up to 9 loci.

SEP procedure

This procedure was performed based on the method of PEP by Zhang, (1992) and is as follows. To the 50 µl PEP mastermix (40 µM 15 bp random primers [Operon Technologies, Alameda, CA], 1X K^+ free PCR buffer [2.5 mM $MgCl_2$; 10 mM Tris.HCl, pH 8.3, gelatin 0.1mg/ml], 83 µM each dNTP, 5U *Taq* polymerase [Perkin Elmer]) was added 10 pmol of each external locus specific primer (5'-ACTTGTCTCCCACTGTTGACT-3'; 5'-TCTTAAAGCATAGGTCATGTG-3';5'-TGAAATAATGGAGATGCAATGTTC-3';5'-GGTCAGGATTGAAAGTGTGCA-3';5'-CAGTGTGAAACGGGGAGAAAACAGT-3'). The mixture is then added to single cell lysates or mock lysates as described above. A mineral oil overlayer (3 drops) was added and tubes were placed into a 96 °C pre-heated PCR machine for cycling. Cycling conditions were 92 °C for 5 min, 32 °C for 90 sec, 55 °C for 2 min followed

by 49 cycles of 92 °C for 30 sec, 37 °C for 90 sec and 55 °C for 30 sec (with fast ramping).

CGH

An aliquot of the PEP plus specific primer PCR mix (ie the SEP mix) was removed and CGH was performed as outlined under the heading 'CGH' in Example 1.

Locus specific analysis

An aliquot of the PEP plus specific primer PCR mix (ie the SEP mix) was then removed to perform individual locus specific PCR reactions. The heminested or fully nested PCR reaction is identical to that performed for any conventional PCR amplification (ie using standard conditions).

CFTR Gene

PCR amplification of exon 11 of the CFTR gene was performed as follows. 2.5 µl SEP aliquots were added to exon 11 mastermix (47.5 µl) containing 10 pmol forward primer, 10 pmol reverse primer (5'-CAACTGTGGTAAAGCAATAGTGT-3'; 5'-GCACAGATTCTGAGTAACCATAAT-3'), 1X PCR buffer [50 mM KCl, 10 mM Tris.HCl, pH 8.3], 100 µM each dNTP, 2.5 mM MgCl and 1U *Taq* polymerase [Perkin Elmer]. Tubes were placed into a pre-heated 96 °C MJ Research PTC-100 PCR machine with hot bonnet and after an initial denaturation step of 94°C for 5 min, cycling conditions were 94°C for 30 sec, 60 °C for 60 sec, 72 °C for 45 sec for 35 cycles.

PCR amplification of exon 4 and 10 was the same as for exon 11 except that 200 µM each dNTP was used instead of 100 µM and primers were for exon 4 5'-AGTTTCACATATGGTATGACCCTC-3' and 5'-TTACTTGTACCAGCTCACTACCTA-3' and for exon 10 5'-AATGATGATTATGGGAGAACTGGAG-3' and 5'-TTCACAGTAGCTTACCCATAGAGG-3'. Cycling conditions were also the same except that 45 cycles was used instead of 35 cycles.

PCR amplification of exon 20 of the CFTR gene followed the procedure of Avner *et al.*, (1994). Briefly, 2.5 µl SEP aliquots were added to mastermix (47.5 µl) containing 10 pmol forward primer, 10 pmol reverse primer (5'-TACCTATATGTCACAGAAGT-

3'; 5'-GTACAAGTATCAAATAGCAG-3'), 1X PCR buffer [50 mM KCl, 10 mM Tris.HCl, pH 8.3], 200 µM each dNTP, 1.5 mM MgCl and 1U *Taq* polymerase [Perkin Elmer]). Tubes were placed into a pre-heated 96 °C MJ Research PTC-100 PCR machine and after an initial denaturation step of 94°C for 5 min, cycling conditions were 94°C for 30 sec, 50 °C for 30 sec, 72 °C for 30 sec for 35 cycles.

SRY Gene

Amplification of the SRY gene was a modification of the method of Cui *et al.*, (1994). A 2.5 µl SEP aliquot was added to 37.5 µl of SRY PCR mix (1X PCR buffer [50 mM KCl, 10 mM Tris.HCl, pH 8.3], 375 µM each dNTP, 3.75 mM MgCl, 10 pmol forward primer (5'-CATGAACGCATTCATCGTGTGGTC-3'; 5'-CTGCGGGAAGCAAACTGCAATTCTT-3'), 10 pmol reverse primer and 1U *Taq* polymerase [Perkin Elmer]). Tubes were placed into a pre-heated 96°C Corbett FTS-100 PCR machine and after an initial denaturation step of 94°C for 5 min, 35 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 2 min were carried out followed by a final extension step of 72°C for 10 min.

PCR products were electrophoresed on 2% agarose gels prestained with ethidium bromide.

Results

Bands were present in all locus specific reactions (exon 4, 10, 11, 20 and the SRY gene) and the intensities were consistent between single cells especially compared to the positive controls. This demonstrates the success of using specific primers in combination with a general (DOP, PEP or similar) PCR primer mix for the amplification of specific loci of particular importance. Thus we have shown from 4 different individual single cells that the invention is able to successfully provide the amplification of 5 loci from a single cell (namely exon 4, 10, 11, and 20 from the CF gene and the SRY gene). This enables the analysis of mutations present in any of these exons of the CF gene (if they were present) and simultaneous gender determination by the presence (male) or absence (female) of a band from the SRY gene PCR reaction.

It is understood that shown in this example is the fact that general primers such as (DOP, PEP and others) are able to be combined successfully with locus specific primers to generate specific analyses as well as enabling the removing an aliquot from

the reaction to perform CGH as described earlier. The PEP reaction used here gives some CGH results although these were not as reliable as CGH that is performed with DOP-PCR general primers. Hence the preference for DOP-PCR primers over PEP primers for general amplification for the purposes of CGH.

DMD Gene

SEP was also performed using Duchenne muscular dystrophy (DMD) as the disease example. The SEP reaction was performed as described for the CFTR gene except that the DMD gene primers for exons 17, 19, 44, 45, 48 and exon 8 (Hussey *et al.*, in press, and references cited therein) were used instead of the CF gene specific primers. All other experimental details were the same. The gel showed the presence of bands in all locus specific reactions (exons 8, 17, 19, 44, 45, 48 from the DMD gene and the SRY gene) and consistent intensities between single cells especially compared to the positive controls. This demonstrates the success of using specific primers in combination with a general (DOP, PEP or similar) PCR primer mix for the amplification of specific loci of particular importance. In addition it demonstrates the universal nature of this invention and the fact that it has a fairly low failure rate.

The presence of amplification products for all 7 loci from 3 different individual single cells demonstrates that the invention is not limited to certain regions of the genome but that it is more widely applicable. The results also demonstrate that this invention is capable of high amplification efficiency as the loci in the latter example are located on the X chromosome and since the single male cell only contains one X chromosome the presence of bands at all loci in all cells demonstrates a low failure rate. If failure rate was high eg caused by allele drop out then we would have expected to see that certain exons have failed to amplify.

One of the benefits flowing from this invention include the fact that karyotyping can be achieved with only minute samples. Thus a single cell can be used as the basis of a DNA sample for testing of chromosomal abnormalities and any specific gene defects of interest for the purposes of prenatal diagnosis and/ or Preimplantation Genetic Diagnosis (PGD).

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CLAIMS

1. A method of comparing at least one chromosome or part thereof from a cell of an individual with an unknown karyotype with the corresponding chromosome or part thereof of an individual with a known karyotype, the method including the steps of :

a. obtaining DNA derived from a single cell of the unknown karyotype,

b. amplifying the chromosomal DNA of the unknown karyotype sufficiently for comparative purposes,

c. labelling the amplified unknown chromosomal DNA with a first label and labelling a known chromosomal DNA with a second label, the first and second label being detectably different,

d. hybridising the labelled known chromosomal DNA with a chromosomal spread, and hybridising the labelled and amplified unknown chromosomal DNA with the chromosomal spread, and

e. mapping the chromosomal spread, and comparing the relative amount of first and second label as a function of position on the at least one chromosome or part thereof.

2. The method of claim 1 wherein the known chromosomal DNA is obtained from a single cell source of known karyotype, and amplified before labelling in step c.

3. The method of claim 2 wherein the single cell source of DNA of known karyotype is white blood cells

4. The method of claim 2 wherein the known chromosomal DNA and the unknown chromosomal DNA are amplified by polymerase chain reaction using the same complement of primers for both.

5. The method of claim 4 wherein the amplification of known and unknown chromosomal DNA is by means of using degenerate primers.

6. The method of claim 4 wherein the primer amplifies a proportion of the genome sufficient to allow for the comparative analysis.

7. The method of claim 4 wherein the amplification method is selected from the list of polymerase chain reaction techniques including DOP-PCR, PEP-PCR, ligation mediated PCR, t-PCR and alu-PCR.

8. The method of claim 7 wherein the amplification method is DOP-PCR.

9. The method of claim 4 wherein the amplified single cell DNA of unknown karyotype is labelled with SpectrumGreen (SG)-dUTP and the DNA of known karyotype is labelled with TRITC.

10. The method of claim 2 wherein the chromosomal spread is a metaphase spread slide from a known normal male.

11. The method of claim 2 wherein the chromosomal spread is an array of different nucleic acids representative of a complement of chromosomes or part thereof and attached to the surface of a substrate.

12. The method of claim 10 or claim 12 wherein greater than 90 % of the distributions of standard deviations in autosomal regions fall within the range 0.85 - 1.15.

13. The method of claim 2 wherein the known and unknown amplified and labelled DNA preparation is hybridised to more than one chromosome spread.

14. The method of any one of claims 1 to 13 wherein the method includes the further step of assessing the number of copies of a chromosome(s) or portion thereof between individuals of known and unknown karyotype.

15. The method of any one of claims 1 to 14 wherein the single cell of unknown karyotype is a prenatal cell and the method is used for the prenatal diagnosis of chromosome differences.

16. The method of claim 15 wherein the prenatal cell is selected from the list including embryo cell, foetal blood cell, oocyte, polar body and sperm cell.

17. The method of claim 16 wherein the prenatal cell is a foetal red blood cell.

18. The method of claim 1 wherein the method is used in diagnosis of gross chromosomal differences and aneuploidy.

19. The method of claim 18 wherein the conditions detectable by the technique include Trisomy 21, 13, 18 and Turner's syndrome (45XO).

20. The method of claim 1 wherein the chromosome spread does not include all chromosomes.

21. The method of claim 1 wherein the known and unknown chromosomal DNA are each isolated from more than one cell, the known and unknown chromosomal DNA each being obtained from the same number or similar number of cells.

22. The method of claim 21 wherein the known and unknown chromosomal DNA are each obtained from less than 10 cells.

23. A method of comparing at least one chromosome or part thereof from a cell of an individual with an unknown karyotype with the corresponding chromosome or part thereof of an individual with a known karyotype, the method including the steps of :

a. obtaining DNA derived from a single cell of the unknown karyotype,

b. amplifying the chromosomal DNA of the unknown karyotype sufficiently for comparative purposes using:

a degenerate primer for amplifying substantially the whole genome, and

at least one specific highlight primer or primer group for amplifying specific loci or loci groups,

c. labelling the amplified unknown chromosomal DNA with a first label and labelling a known chromosomal DNA with a second label, the first and second label being detectably different,

d. hybridising the labelled known chromosomal DNA with a chromosomal spread, and hybridising the labelled and amplified unknown chromosomal DNA with the chromosomal spread,

e. mapping the chromosomal spread, and comparing the relative amount of first and second label as a function of position on the at least one chromosome or part thereof,

and

f. analysing specific loci to determine allele type.

24. The method of claim 23 wherein the method is used to detect general chromosomal abnormalities as well as allele differences for specific loci.

25. The method of claim 24 wherein the highlight primer binds to sequences known to be present in certain positions of the target for highlighting.

26. The method of claim 25 wherein the DNA sequences for a chromosome to be highlighted is determined from the data resulting from the cooperative project for DNA sequencing of the entire human genome known as HUGO.

27. The method of claim 24 wherein the specific loci are selected from the list including those for cystic fibrosis, thalassemia, Duchenne muscular dystrophy, rare X-linked disorders, Haemophilia and Huntington's Disease.

28. The method of claim 24 wherein the specific loci are polymorphic loci and the method enables paternity/ maternity testing.

29. A kit comprising reagents for performing the method of any of claims 1 to 28.

AMENDED CLAIMS

[received by the International Bureau on 02 February 2000 (02.02.00);
original claim 2 cancelled; original claim 1 amended;
claims 2-29 renumbered as claims 2-28 (4 pages)]

1. A method of comparing at least one chromosome or part thereof from a cell of an individual with an unknown karyotype with the corresponding chromosome or part thereof of an individual with a known karyotype, the method including the steps of :

a. obtaining DNA derived from a single cell of the unknown karyotype,

b. amplifying the chromosomal DNA of the unknown karyotype sufficiently for comparative purposes,

c. labelling the amplified unknown chromosomal DNA with a first label and labelling a known chromosomal DNA obtained and amplified from a single cell source of known karyotype with a second label, the first and second label being detectably different,

d. hybridising the labelled known chromosomal DNA with a chromosomal spread, and hybridising the labelled and amplified unknown chromosomal DNA with the chromosomal spread, and

e. mapping the chromosomal spread, and comparing the relative amount of first and second label as a function of position on the at least one chromosome or part thereof.

2. The method of claim 1 wherein the single cell source of DNA of known karyotype is white blood cells

3. The method of claim 1 wherein the known chromosomal DNA and the unknown chromosomal DNA are amplified by polymerase chain reaction using the same complement of primers for both.

4. The method of claim 3 wherein the amplification of known and unknown chromosomal DNA is by means of using degenerate primers.

5. The method of claim 3 wherein the primer amplifies a proportion of the genome sufficient to allow for the comparative analysis.

6. The method of claim 3 wherein the amplification method is selected from the list of polymerase chain reaction techniques including DOP-PCR, PEP-PCR, ligation mediated PCR, t-PCR and alu-PCR.

7. The method of claim 6 wherein the amplification method is DOP-PCR.

8. The method of claim 3 wherein the amplified single cell DNA of unknown karyotype is labelled with SpectrumGreen (SG)-dUTP and the DNA of known karyotype is labelled with TRITC.

9. The method of claim 1 wherein the chromosomal spread is a metaphase spread slide from a known normal male.

10. The method of claim 1 wherein the chromosomal spread is an array of different nucleic acids representative of a complement of chromosomes or part thereof and attached to the surface of a substrate.

11. The method of claim 9 or claim 10 wherein greater than 90 % of the distributions of standard deviations in autosomal regions fall within the range 0.85 - 1.15.

12. The method of claim 1 wherein the known and unknown amplified and labelled DNA preparation is hybridised to more than one chromosome spread.

13. The method of any one of claims 1 to 12 wherein the method includes the further step of assessing the number of copies of a chromosome(s) or portion thereof between individuals of known and unknown karyotype.

14. The method of any one of claims 1 to 13 wherein the single cell of unknown karyotype is a prenatal cell and the method is used for the prenatal diagnosis of chromosome differences.

15. The method of claim 14 wherein the prenatal cell is selected from the list including embryo cell, foetal blood cell, oocyte, polar body and sperm cell.

16. The method of claim 15 wherein the prenatal cell is a foetal red blood cell.

17. The method of claim 1 wherein the method is used in diagnosis of gross chromosomal differences and aneuploidy.

18. The method of claim 17 wherein the conditions detectable by the technique include Trisomy 21, 13, 18 and Turner's syndrome (45XO).

19. The method of claim 1 wherein the chromosome spread does not include all chromosomes.

20. The method of claim 1 wherein the known and unknown chromosomal DNA are each isolated from more than one cell, the known and unknown chromosomal DNA each being obtained from the same number or similar number of cells.

21. The method of claim 20 wherein the known and unknown chromosomal DNA are each obtained from less than 10 cells.

22. A method of comparing at least one chromosome or part thereof from a cell of an individual with an unknown karyotype with the corresponding chromosome or part thereof of an individual with a known karyotype, the method including the steps of :

a. obtaining DNA derived from a single cell of the unknown karyotype,

b. amplifying the chromosomal DNA of the unknown karyotype sufficiently for comparative purposes using:

a degenerate primer for amplifying substantially the whole genome, and

at least one specific highlight primer or primer group for amplifying specific loci or loci groups,

c. labelling the amplified unknown chromosomal DNA with a first label and labelling a known chromosomal DNA with a second label, the first and second label being detectably different,

d. hybridising the labelled known chromosomal DNA with a chromosomal spread, and hybridising the labelled and amplified unknown chromosomal DNA with the chromosomal spread,

e. mapping the chromosomal spread, and comparing the relative amount of first and second label as a function of position on the at least one chromosome or part thereof,
and
f. analysing specific loci to determine allele type.

23. The method of claim 22 wherein the method is used to detect general chromosomal abnormalities as well as allele differences for specific loci.

24. The method of claim 23 wherein the highlight primer binds to sequences known to be present in certain positions of the target for highlighting.

25. The method of claim 24 wherein the DNA sequences for a chromosome to be highlighted is determined from the data resulting from the cooperative project for DNA sequencing of the entire human genome known as HUGO.

26. The method of claim 23 wherein the specific loci are selected from the list including those for cystic fibrosis, thalassemia, Duchenne muscular dystrophy, rare X-linked disorders, Haemophilia and Huntington's Disease.

27. The method of claim 23 wherein the specific loci are polymorphic loci and the method enables paternity/ maternity testing.

28. A kit comprising reagents for performing the method of any of claims 1 to 27.

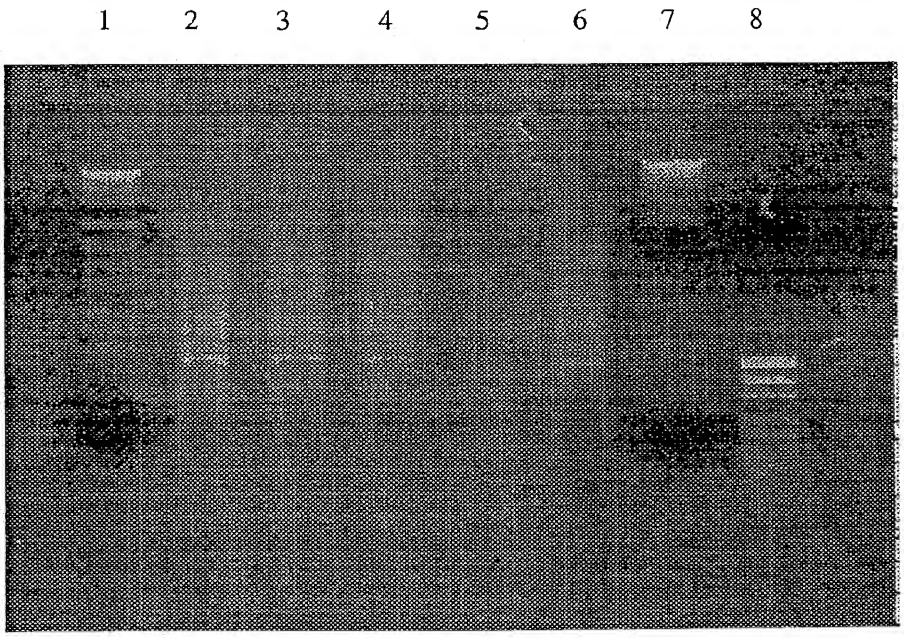


FIGURE 1

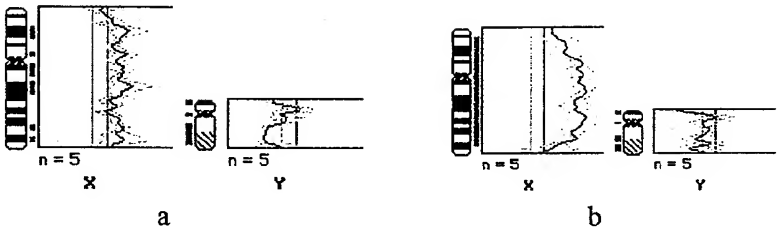


FIGURE 2

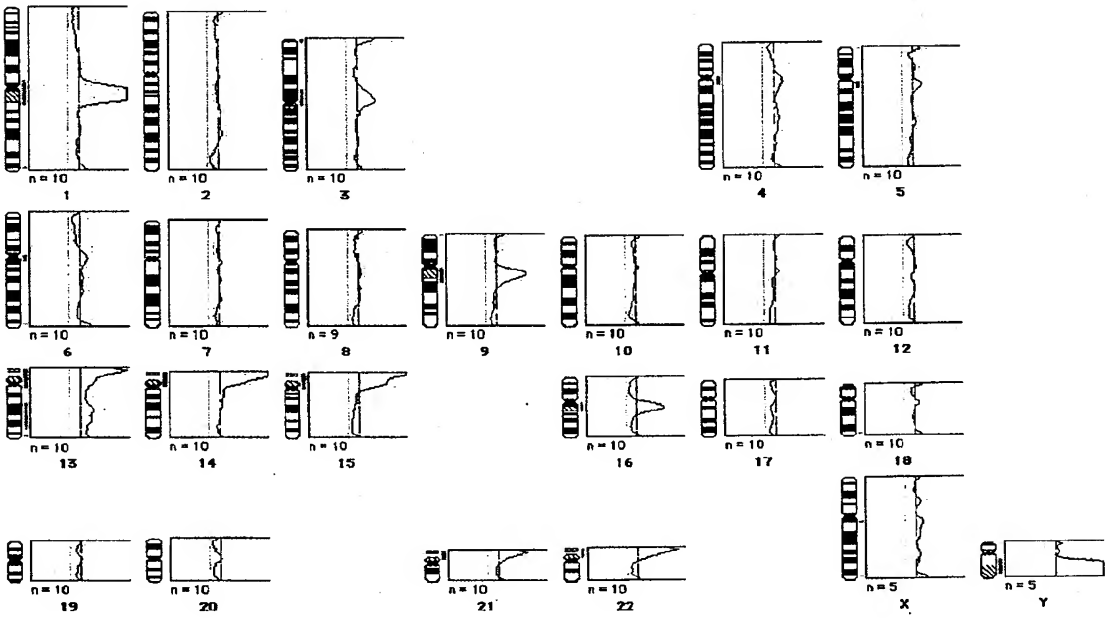
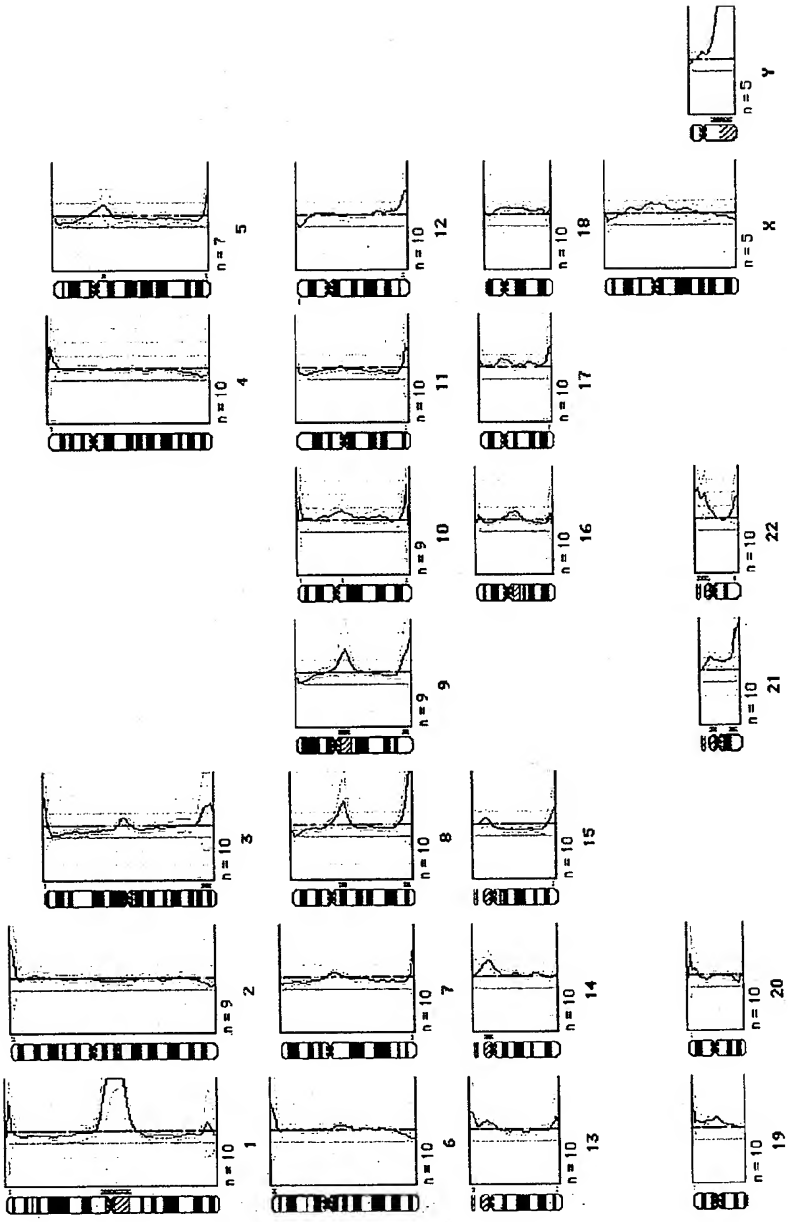


FIGURE 3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n=10 14
n=10 15
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n=10 22
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n=5 Y

FIGURE 4

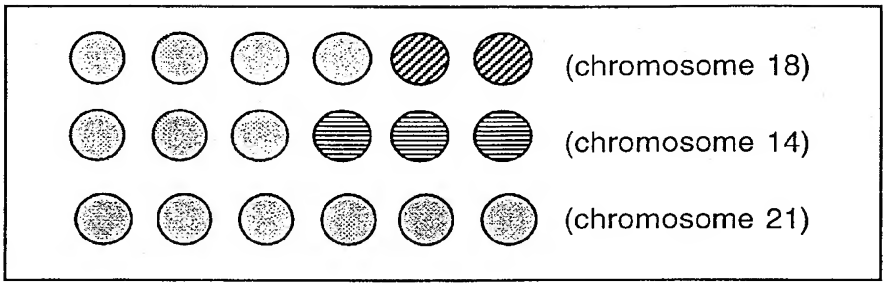

FIGURE 5

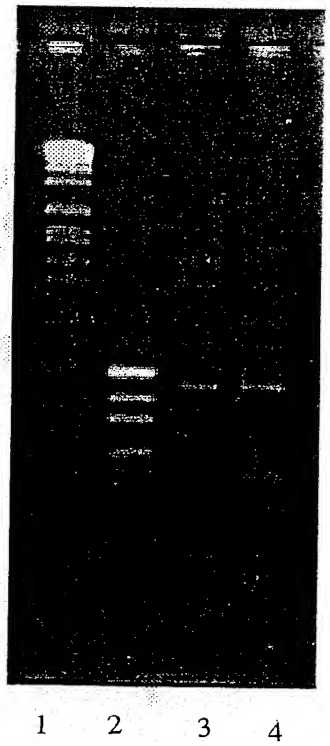

FIGURE 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00938

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12Q 001/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
AS ABOVE

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AS BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, Chem Abs, Medline: -
DOP/degenerate oligo primed; PCR/polymerase chain reaction; chromosom/karyotyp; single (cell/copy/target)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Hussey et al. Molecular Human Reproduction. 1999. 5(11): 1089-1094. Whole document	1-29
X	Kuukasjarvi et al. Genes Chromosomes and Cancer. 1997. 18: 94-101. Whole document	1-29
X	Engalen et al. Clinical Genetics. 199649: 242-248. Whole document	1-29

☐ Further documents are listed in the continuation of Box C

☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25/11/99	10 DEC 1999
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer **GILLIAN ALLEN** Telephone No.: (02) 6283 2266

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00938

C (Continuation). **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Muller-Navia et al. Human Genetics. 1995. **96**: 661-667. Whole document	1-29
Y	Cheung et al. Proc Nat Acad Sci USA. 1996. Whole document	1-29
A	Telenius et al. Genetics. 1992. 718-725. Whole document	1-29